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(54) Title: IMPROVEMENTS IN OR RELATING TO GERMICIDAL COMPOSITIONS

(57) Abstract

A surface germicidal composition comprises a dyestuff which is capable of photo-dynamic inactivation of micro-organisms. The dyestuff preferably generates singlet oxygen on exposure to light and is substantive to microorganisms, conveniently being selected from the group comprising Rose Bengal, Erythrosin B and phthalocyanin sulphonates. The composition may optionally include other ingredients such as one or more surfactants (for cleaning purposes) and/or one or more solvents.



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Title: Improvements in or Relating to Germicidal Compositions

## Field of the Invention

This invention relates to germicidal compositions, particularly for use on surfaces, ie compositions capable of destroying or inactivating micro-organisms, particularly surface-bound micro-organisms.

## Summary of the Invention

In its broadest aspect the present invention provides a surface germicidal composition comprising a dyestuff which is capable of photo-dynamic inactivation of micro-organisms.

It is preferred to use a dyestuff that generates singlet oxygen on exposure to light. On absorption of light energy a dye molecule is converted to a more energetic or exited state ($S_1^*$) from its electronic ground state ($S_0$). (Electron spins are paired and so these are singlet states in the language of spectroscopy, having a single energy level in a magnetic field.)

The excited state is short lived and can lose energy and return to the ground state in a number of ways: by emission of a quantum of light as fluoresecence; by internal conversion as the energy is degraded to heat; by collision with a molecule of a different substance

(fluorescence quenching).

The short lived singlet state may also undergo a process called intersystem crossing to a longer-lived excited state, the triplet state. (The state is termed "triplet" because the electron in the higher energy level is no longer spin-paired with the electron in the lower level and the excited state has three energy levels in a magnetic field.)

Interaction of the excited triplet state with ground state molecular oxygen (which exists in the triplet state normally) regenerates the dye ground state as energy is transferred to the oxygen which is promoted to the electronically excited singlet state. This means that, under ideal circumstances, a single photosensitiser molecule can generate many times its own concentration of singlet oxygen.

Singlet oxygen is highly reactive and photosensitised oxidation proceeding via this route is known as Type II photo-oxidation. Type II photo-oxidation is independent of the photosensitiser used to generate the singlet oxygen. An important feature of the sensitiser is that it should have a high quantum yield of triplet formation (that is, ideally, a triplet state should be produced for each photon absorbed). Intersystem crossing to the triplet state is facilitated by the presence of heavy atoms in the molecule.

Photo-oxidation of any vital component of an organism may result in cell death (protein, polypeptide, amino-acids, lipids with allylic hydrogens, tocopherols, sugars and cellulose).

Currently preferred dystuffs include Rose Bengal (Acid Red 94, Colour Index No. 45440), Erythrosin B (Acid Red 51, Colour Index No. 45430), and phthalocyanin sulphonates such as aluminium phthalocyanin sulphonate (APS) and zinc phthalocyanin sulphonate (ZPS). Rose Bengal and Erythrosin B are known food colourants (Rose Bengal is Food Colour Red No 105 and Erythrosin B is Food Colour Red No 14), and Erythrosin B is on an EEC list of colouring agents allowed for use in cosmetic products, so these two dyes are well suited for use in compositions intended for domestic use. Mixtures of dyes can be used, and in some cases it may be desirable to include in a mixture a dye that will remain visible at the end of the photodynamic process.

The concentration of dyestuff in the composition is not critical and will typically be up to 100ppm, with good results having been obtained with concentrations in the range 10ppm to 20ppm. Lower concentrations, down to 1ppm should also give reasonable results.

Singlet oxygen has a short lifetime and therefore a short pathlength for diffusion, so to be effective a photosensitising dye generating singlet oxygen must be close to the target substrate. Preferred dyestuffs are therefore substantive to (ie capable of binding to) micro-organisms, typically by binding to cellular protein on the organism surface or other cellular components eg cellular fats.

The preferred dyes mentioned above generate singlet oxygen on exposure to light and are substantive to protein and so capable of binding to micro-organisms via cellular protein. In this way, targetted killing of organisms and

hence germicidal action is possible.

It is also preferred to use a dyestuff that is bleached by exposure to light. By use of a photo-bleaching dyestuff that is substantive to micro-organisms it can be possible for a visible indication of the presence of micro-organisms to be provided. As the dyestuff bleaches, the photo-dynamic action proceeds causing the death of, or otherwise inactivating, micro-organisms. In the presence of low levels of light, both bleaching and the photo-dynamic activity are believed to proceed more slowly, whereas at higher light intensities both processes occur more quickly. Thus, depending on the relative rates of bleaching and photo-dynamic activity, the presence of visible dyestuff indicates to the user that the photo-dynamic inactivation of any micro-organisms present is incomplete.

The photo-dynamic inactivation of micro-organisms in suspension by dyes such as Rose Bengal is known. See, for example, Journal of Applied Bacteriology 1985, [58] pages 391-400, Photochemistry and Photobiology 1988, [48] pages 607-612 (Neckers et al), and Shokuhin Eiseigaku Zasshi 1962, 10(5), pages 344-347. However, it has now been found that suitable dyestuffs are capable of photo-dynamic inactivation of micro-organisms on surfaces, and this is the basis of the present invention. It is well-known that micro-organisms are much more susceptible to biocides in their planktonic or suspended state: they are much more difficult to inactivate when attached to surfaces, which is their usual or preferred state. Micro-organisms will normally be on surfaces in the form of "biofilms", that is, embedded in a matrix of extracellular material. This extracellular material may sometimes be referred to as

"adhesin" in the literature. It is therefore not obvious that a process which acts on micro-organisms in their planktonic state would act on surface-bound organisms without modification being required. Surface-bound micro-organisms represent an important and substantial source of contamination in domestic, institutional and industrial environments, and the present invention can enable targetted germicidal action on such micro-organisms.

Compositions according to the present invention are particularly suitable for use on hard domestic and industrial surfaces such as glass, plastics, ceramic and metal surfaces. In particularly, the compositions are effective for use on surfaces which may harbour soils having the potential for bacteriological contamination in surface imperfections, joints and other relatively confined regions.

The composition is preferably acidic, eg having a pH in the range of 3 to 5, eg a pH of about 4, as acidic compositions are found to have substantially enhanced effectiveness against Gram-negative (G-) micro-organisms as compared with neutral compositions. The effectiveness against Gram-positive (G+) micro-organisms seems not to be significantly affected by pH. The compositions are conveniently made acidic by use of relatively mild organic acid, such as acetic acid.

Neckers et al (above) review conflicting evidence that penetration of the dye Rose Bengal itself through the cell wall is essential for inactivation. They suggest that their own results support the dye penetration hypothesis primarily on the basis of differential inactivation of G+ and G- species. The envelope of G- bacteria has an

additional outer membrane composed substantially of lipopolysaccharide which Necker et al regard as serving as a barrier to potentially toxic substances. An alternative interpretation could be that the amount of protein exposed in the cell walls is very different between G+ and G- species, being greater for G+ than G-, with a binding affinity for the dye which varies with pH. The concentration of dye bound to the cell wall would therefore be a function of pH. This interpretation would account for our observations of the variation in kill rate with pH (but not the apparent resistance of the G+ species B. subtilis and B. megaterium).

There is no difference between the endospore-forming species B. subtilis and the nonspore-forming S. aureus in their susceptibilities to photodynamic action by Rose Bengal in the absence of endospores. The apparent differences shown in our studies are presumably due to the presence of endospores both for B. subtilis and B. megaterium which are clearly more resistant to singlet oxygen than the bacteria themselves. The spores survive exposure to Rose Bengal and light and subsequently germinate to give countable colonies. Spores are known to be difficult to stain and presumably have no affinity for Rose Bengal under any of the conditions used. There appears to be little documentation on the toxicity of singlet oxygen to spores in the literature. From studies on the possibly less resistant conidia of Neurospora crassa (Photochem. Photobiol., 33, 349 (1981)), singlet oxygen does have potential in this respect.

The composition may optionally include other ingredients such as one or more surfactants (for cleaning purposes) and/or one or more solvents.

The surfactant is preferably alkoxylated, more preferably ethoxylated, eg being in the form of ethoxylated alcohols. The alcohol preferably has between 4 and 15 carbon atoms, is of straight or branched chain configuration, and has an HLB value (hydrophilic lipophilic balance) in the range 10 to 14, eg 12.

A wide range of suitable surfactants are commercially available, one such material being the surfactant available under the trade name Imbentin 91-35, from Kolb, which is a nonionic C9-11 alcohol ethoxylate, having an average of 5 moles of ethylene oxide per mole of alcohol.

Primary ethoxy sulphates may also be used.

Mixtures of surfactants may be used if desired.

The surfactant is preferably non-ionic or anionic, or a mixture of both types.

Preferred anionic surfactants for this purpose include primary alkyl sulphates (PAS), preferably sodium dodecyl sulphate (SDS). Commercial mixtures containing a substantial proportion of dodecyl sulphate (eg Empicol LX) are espcially preferred. Dodecyl sulphate is a known protein denaturant, is good for cleaning protein off surfaces, and is biocidal.

The composition is preferably substantially free of cationic surfactant, but may include a minor amount of cationic germicide.

Surfactant preferably constitutes an amount in the range

0.05 to 2.5% by weight of the total weight of the composition, typically 0.5% to 1.5% by weight, eg 0.7% by weight nonionic surfactant with an optional amount of up to 0.2% by weight of anionic surfactant.

The solvent is preferably polar and is preferably a straight or branched chain C2 to C5 alcohol such as ethanol, butanol, isopropanol (propan-2-ol) (IPA), N-butoxy propan-2-ol (propylene glycol n-butyl ether), 2-butoxy ethanol (ethylene glycol monobutyl ether). IPA is the currently preferred solvent.

Dihydric alcohol such as ethylene glycol, and water miscible ethers such as dimethoxyethane may also be used.

Mixtures of solvents can be used if appropriate, eg mixtures of ethanol and N-butoxy propan-2-ol.

Solvent is preferably present in an amount in the range 2 to 20% by weight of the total weight of the composition.

At least some of these solvents, eg ethanol, weaken the cell walls of micro-organisms, making them more permeable and so more susceptible to penetration by singlet oxygen. This has the effect of enhancing the micro-organism-killing effect of the dyestuff.

It is found that inclusion of surfactant can reduce the photo-dynamic effect of dyestuffs (possibly by solubilising the dyestuff and preventing adsorption on the cell wall), and inclusion of solvent can also reduce the photo-dynamic effect of dystuffs (possibly by competing for singlet oxygen). However, it is found that with three component formulations, comprising dyestuff, surfactant

and solvent, the reduction in photo-dynamic effect of the dyestuff is less than would be expected from the combined effects of surfactant and solvent. The surfactant and solvent together thus have a synergistic effect, the result of which is to lower the reduction in the photo-dynamic effect of the dyestuff.

In a preferred aspect, the present invention thus provides a surface cleaning and germicidal composition, comprising a dyestuff which is capable of photo-dynamic inactivation of micro-organisms, a surfactant and a solvent.

The composition may include a number of optional ingredients including the following:

1. Detergent boosters, preferably metal chelating agents such as ethylene diamine tetra acetic acid (EDTA). Metal chelating agents (including EDTA) have also been claimed to permeabilise cell walls, thus making organisms more susceptible to biocides.

2. Electrolyte such as a buffer or salt, eg $Na_2SO_4$, which acts to assist binding of dye to protein by promoting movement of dye from the aqueous phase to the protein salt. Electrolyte is commonly present in various dye formulations as commercially available, although additional electrolyte can be added if required. Total electrolyte content of the composition would typically be in the range 0 to 1% by weight, preferably about 0.1%.

3. Perfumes.

4. Thickeners.

The composition is in the form of an isotropic, single phase composition and is of particular use as a germicide (possibly also with a cleaning effect) on hard surfaces, finding application in a wide range of contexts, including domestic applications, eg kitchen and bathroom surfaces including toilet bowls, in institutions such as schools, hospitals etc, and in commercial premises such as factories, offices, hotels etc.

For domestic use at least, the composition is preferably formulated as a product intended for application by spraying and is conveniently packaged in a suitable container, eg having a hand operated trigger spray or an aerosol propellant dispenser. The container is preferably light-opaque.

In use, the composition is applied to a surface to be treated in any convenient manner, eg by spraying from a suitable dispenser, wiping on with a carrier such as a cloth or sponge, or pouring from a container etc. This might in some cases, particularly in industrial cleaning, be followed by exposure to a light source, eg a white light source such as a quartz halogen lamp or fluorescent "daylight" source. (The process would be an alternative to using dangerous germicidal radiation, for example from a low pressure mercury discharge lamp emitting resonance radiation at 254 nm. Such radiation is harmful to the unprotected eye.) This would generally be followed by a rinsing step, if required, eg by wiping with a carrier, application of a stream of running water etc.

In a further aspect, the invention thus provides a method of killing bacteria on a surface, comprising applying to the surface a composition in accordance with the

invention.

The invention will be further described, by way of illustration, in the following Examples and by reference to the accompanying Figures in which:

Figure 1 shows two bar charts of log (reduction) values illustrating the lethal effect of Rose Bengal and light on various micro-organisms in suspension at pH4 and pH7, with Figure 1a showing results for Gram-positive micro-organisms, and Figure 1b showing results for Gram-negative organisms and yeasts;

Figure 2 is a pair of graphs of log (reduction) versus pH showing the biocidal effect of Rose Bengal and light on S. aureus and E. coli as a function of pH, with Figure 2a showing results after 20 minutes exposure to light and Figure 2b showing results after 60 minutes exposure;

Figure 3 is a pair of graphs similar to Figure 2 obtained using Erythrosin B in place of Rose Bengal;

Figure 4 shows two bar charts of log (reduction) illustrating the photohygiene effect of various combinations of Rose Bengal (RB), Imbentin C91-35 (AE), isopropranol (IPA) and Empicol LX (PAS), with Figure 4a showing results obtained without exposure to light and Figure 4b showing results obtained with exposure to light;

Figure 5 is a graph of adsorption of Rose Bengal by E. coli, with amount adsorbed (nanomoles) versus equilibrium concentration (micromoles/l), with results at pH 4 shown by squares and results at pH 7 shown by crosses;

Figure 6 is a pair of graphs similar to Figure 5 showing the effect of electrolyte, with Figure 6a showing results at pH 4 and Figure 6b showing results at pH 7, with results without additive shown by squares, results with sodium sulphate (1%) shown by crosses and results with sodium sulphate (5%) shown by double crosses;

Figure 7 is a pair of graphs similar to Figure 5 showing the effect of surfactant, with Figure 7a showing results at pH 4 and Figure 7b showing results at pH 7, with results without additive shown by squares, results with non-ionic surfactant (0.7%) (NI) shown by crosses and results with PAS (0.7%) shown by double crosses;

Figure 8 is a graph similar to Figure 5 showing the effect of solvent at pH 4, with results without additive shown by small squares, those for 10% IPA shown by crosses, those for 0.7% Imbentin shown by double crosses and those for 10% IPA and 0.7% Imbentin shown by large squares;

Figure 9 is a graph of log (reduction) versus exposure time (minutes) showing the effectof pH on the rate of kill of E. coli by Rose Bengal, with results at pH 4 shown by squares and results at pH 7 shown by crosses; and

Figure 10 is a graph similar to Figure 9 showing the effect of electrolyte at pH 7 on the rate of kill of E.coli by Rose Bengal, with results without electrolyte shown by squares and results with Na sulphate shown by crosses.

Examples

EXPERIMENTAL PROCEDURES

Preparation of Inocula

The following micro-organisms (generally either from the National Collection of Type Cultures (NCTC) or the American Type Culture Collection (ATCC)) were used in the experiments described:

Bacteria:

| | | |
|---|---|---|
| Staphylococcus aureus | NCTC 6538 | (Gram positive) |
| Escherichia coli | NCTC 8196 | (Gram negative) |
| Pseudomonas aeruginosa | NCTC 5940 | (Gram negative) |
| Enterobacter sp. | NCTC 3281 | (Gram negative) |
| Klebsiella sp. | ATCC 11677 | (Gram negative) |
| Bacillus subtilis | NCTC 6432 | (Gram positive) |
| Bacillus megaterium | NCTC 7581 | (Gram positive) |

Yeast:

Candida albicans

Organisms were grown up by overnight incubation in nutrient broth at 37°C for bacteria (28°C for Ps. aeruginosa) or SABS broth (SABS is Sabourand Dextrose Agar, with liquid medium in the case of SABS broth, from Oxoid Ltd) at 28°C for yeast. Cultures were isolated by vacuum filtration using a 0.45um Millipore filter and washed with quarter-strength Ringers solution before resuspension in Ringers solution (10ml). The organisms in suspension were enumerated by serial dilution and plating with nutrient agar (bacteria) or SABS agar (yeast) and the total viable count (TVC) expressed as the decadic

logarithm of the number of colony-forming units (cfu) per ml.

Experiments were carried out at pH 7 unless otherwise specified.

1) AGAR DIFFUSION DISC METHOD

Aqueous solutions containing 100 ppm of dye were prepared. Aliquots (10ml) of each dye solution were sterilized in glass universal screw cap vials. Antibiotic assay discs (13mm from BDH) were also sterilized. All organisms were grown overnight in nutrient or SABS broth (10ml).

For each micro-organism, two nutrient agar plates (SABS agar for the yeast) were seeded with the overnight culture (10ul) to give confluent growth over the whole plate. Using aseptic techniques, an antibiotic disc was dipped into the first dye solution and placed on the surface of a seeded agar plate. This was repeated with two other dye solutions to give three discs on duplicate plates.

One of each pair of duplicate plates was immediately placed in an incubator at the appropriate temperature with minimal exposure to light. The remaining plates were placed on top of a light box for 3 hours. Illumination from the light box was diffused white light of mean intensity 4000 lux from fluorescent "daylight" tubes (2 x 15 watt, Exal X-ray Accessories Ltd, Hemel Hempstead). Light intensities were measured at the surface of the diffuser using a Megatron DA10 light meter. After exposure the plates were incubated overnight and then examined for zones of inhibition around the disc.

Using this approach, the following results were obtained:

Example 1 (Disc Method)

Results on agar for aqueous dye solutions of Rose Bengal, Erythrosin B and aluminium phthalocyanin sulphonate (APS) (100 ppm) at pH 7 after exposure to light for 180 minutes as described above are summarised in Table 1. In the Table, the results are expressed as the difference (in millimetres) between the radius of the clear zone of inhibition (the area of no bacterial growth on the spread agar plate) and the radius of the disc. Thus, the higher the value, the greater the baterial kill.

The agar diffusion disc method ranked Rose Bengal as more effective than the structurally similar Erythrosin B. It is tempting to ascribe this ranking to a difference in the quantum yield for singlet oxygen formation. In methanol, the quantum yield for singlet oxygen formation is 0.76 for Rose Bengal compared to 0.6 for Erythrosin B. However, a number of other factors might also be expected to contribute to the observed differences such as the rate of dye diffusion or differences in dye binding to the agar gel or disc material.

A particular feature of the results from the disc method is the clear distinction in the sensitivity of Gram-positive (G+) and Gram-negative (G-) organisms to photodynamic action, at least at pH 7. Possible reasons for the relative resistance of G- organisms are discussed elsewhere.

2) SURFACE TEST

Test Solutions

Rose Bengal (20ppm) in pH 4 buffer and:

1. No further addition
2. Ethanol (10 % v/v) and Imbentin C91-35 (0.7%).
3. Propan-2-ol (10% v/v) and Imbentin C91-35 (0.7%).

Sodium hypochlorite solution (0.125 %) was used as a positive control.

Estimation of Number of Organisms Adherent to Base of Petri Dish

Micro-organism, eg S. aureus, suspension (0.5ml) was added to aliquots (100 ml) of quarter-strength Ringers solution and the average cfu per ml determined (TVC). Aliquots (20ml) of these solutions were pipetted into sterile petri dishes and left at room temperature for 5 hours. The inoculum was then removed by pipette into a sterile bottle and the average cfu per ml remaining in suspension estimated. The number of organisms (as cfu) per square cm adherent to the Petri dish was calculated from the difference in the solution concentrations.

Test Method

Bacterial suspensions in quarter-strength Ringers (20mls) were pipetted into sterile plastic Petri dishes which were left at ambient temperature for 5 hours. The inoculum was then removed, the dishes washed once with Ringers solution with gentle swirling by hand, and the solution poured off. Pairs of bacterially contaminated plates were treated with

the test solutions. An aliquot (20ml) of test solution was poured into one dish and the solution decanted after 30 seconds. The dish was rinsed with pH 4 buffer and placed on the light box for 20 mins. In a separate experiment, the solution was exposed in the second dish on the light box for 20 minutes before being decanted and the dish rinsed with pH 4 buffer. Both experiments were duplicated.

After light exposure, one of the duplicate plates was overlaid with Tryptone Soya agar containing 1% glucose and 0.015% Neutral Red cooled to about 50°C. The other duplicate plate was stained with 0.01% Acridine Orange for 30 seconds, rinsed and examined microscopically (Nikon "Optiphot" microscope equipped with a 100x apochromat oil-immersion objective, 10x eyepiece and epifluorescence attachment with a B2-A combined filter/dichroic mirror block and super high pressure mercury lamp). The overlaid agar plates were incubated at 37°C for 48 hours, by which time colonies had grown out of the adherent bacteria which had not been killed. The colonies took up the Neutral Red and could be seen and counted under the agar on the surface of the dish. (A M R MacKenzie and R L Rivera-Calderon, Agar Overlay Method to Measure Adherence of Staphylococcus epidermidis to Four Plastic Surfaces, Applied and Environmental Microbiology, 50, 1322 (1985).)

Plates stained with Acridine orange were examined and photographed. The number of stained bacteria were counted in a field of view (clumps counted as one) which had previously been estimated by photographing a micrometer scale.

Example 2 (Surface Test)

Control experiment surface tests using both direct epifluorescence microscopy as described above and suspension depletion gave similar values for the number of bacteria (S. aureus) that could be attached to the surface of a plastic dish (of the order of one million per square centimetre).

Contracting the surface with the positive control (sodium hypochlorite solution, 30 seconds) reduced the number of viable bacteria to zero. The results for photo-dynamically inactivated bacteria are summarised in Table 2 as the decadic logarithm of the reduction (log (reduction)) of viable bacteria before and after exposure, ie log (start count) - log (final count).

Example 3

Formulation: Rose Bengal (20 ppm), nonionic surfactant (Imbentin C91-35, 0.7%), propan-2-ol (10 %) (pH 4).

Experimental procedure was as previously described except that surface attachment of the bacteria required them to be in the exponential growth phase. This was achieved by incubation in nutrient broth (for bacteria) or SABS broth (for yeast) in the plastic Petri-dish for 3 hours. In Example 2, a suspension of non-growing bacteria in Ringers' solution was simply allowed to stand for 5 hours. This worked well for S. aureus but not other bacteria. The results are shown in Table 3.

Previous experience using direct epifluorescence microscopy suggests that where confluent growth was obtained, this would be equivalent to an initial surface

density of viable microbes of the order of 1 million/sq. cm. The surface area of the Petri-dishes used was about 57 sq.cm. so that the treatment in all cases has reduced the level of surface contamination by at least 5 orders of magnitude (log 5).

Surface tests are more difficult to perform than suspension tests, and for this reason most experimental work has been performed in suspension to demonstrate the effect on varying conditions.

3) SUSPENSION TEST

Preparation of Solutions

Stock solutions of the following were prepared by weighing and sterilized (except those containing solvent):

Rose Bengal (0.2 percent) in propan-2-ol (95 percent
Nonionic surfactant (Imbentin C91-35, 14 percent) (sometimes referred to by the abbreviation AE, for alcohol ethoxylate)
Anionic surfactant (Empicol LX, 14 percent) (sometimes referred to as PAS)
pH 4 buffer (citric acid (0.1 M,307 ml) + dibasic sodium phosphate (0.2 M, 193 ml)
pH 7 buffer (sodium dihydrogen orthophosphate (0.4M,468 ml) + disodium hydrogen orthophosphate dodecahydrate, (0.4M,732 ml)
Buffers pH 5,6,8,9 were prepared as indicated in the CRC Handbook of Chemistry and Physics, 8-36, 73rd Ed., CRC Press (1992-1993)

Final concentrations in the test solutions were

typically:

Rose Bengal - 20ppm
Ethanol - 10.0% (v/v)
Surfactant - 0.7% (w/v)

In some examples, different concentrations were used as specified.

Test Method

Test solutions were made up in sterile plastic petri dishes to a depth of 5mm (30mls). A suspension of micro-organism (0.3ml) was added to each solution and gently mixed in. If Rose Bengal was to be included in the test solution it was added last to minimise light exposure. Solutions were either exposed on a light box, placed in the dark (conditions of reduced light exposure) or left on the bench. The average intensity at the surface of the light box diffuser was 4000 lux measured with a Megatron DA 10 light meter (from Megatron Ltd). After specified exposure times, surviving bacteria were enumerated as colony-forming units (cfu/ml) following incubation after serial dilution and plating onto agar. The decadic logarithm of the number of bacteria remaining (as colony-forming units per ml) was determined and compared to the number before exposure as log (start count) - log (final count). The higher the value, the greater the bacterial kill.

Suspension tests have been carried out against a variety of organisms under a variety of conditions to optimise photo-dynamic action against a range of micro-organisms, including Gram-negative organisms and yeast. Salient

results are summarised in the associated Tables. In these Tables results are expressed as the decadic logarithm of the ratio of the initial number of colony-forming units per ml to the number remaining after exposure, the log (reduction). Using this notation a value of zero means no change in the number of organisms following exposure to the conditions. The notation "+" preceding a log reduction figure indicates that no micro-organism growth could be observed (ie total kill).

Example 4

Suspension tests were carried out as described above to show the lethal effect of Rose Bengal and light on micro-organisms in suspension; particularly the synergy of low pH and ethanol solvent. Tests were carried using Rose Bengal (20ppm) alone or with ethanol (10% (v/v), with light exposure of 20 minutes (light box). The results expressed as log (reduction) values are given in Table 4.

Longer exposure times (of 60 and 100 minutes) improve performance for Gram negative organisms, particularly at pH 7.

It will be seen that performance for Gram negative organisms is much improved at pH 4 as compared with pH 7.

Example 5

Suspension tests were carried out as described above at pH 4 and pH 7 to show the lethal effect of Rose Bengal (20ppm) and light (20 minutes exposure on a light box) of various Gram-positive and Gram-negative micro-organisms and yeast in suspension, and the results expressed as log

(reduction) are shown graphically in Figures 1a and 1b, with Figure 1a giving results for Gram-positive micro-organisms and Figure 1b giving results for Gram-negative micro-organisms and yeast.

Example 6

Suspension tests were carried out as described above at a range of different pHs to show the biocidal effect of Rose Bengal (20ppm) and light on S. aureus (G+) and E. coli (G-) as a function of pH, and the results expressed as log (reduction) are shown graphically in Figures 2a (exposure time 20 minutes) and 2b (exposure time 60 minutes). In the Figures, crosses show results for control (G-), double crosses show results for E. coli, inverted triangles show results for control (G+) and triangles show results for S. aureus.

Similar suspension tests were carried out to show the biocidal effect of Erythrosin B and light on S. aureus and E. coli as a function of pH, and the results are shown graphically in Figures 3a and 3b, which are otherwise identical to Figures 2a and 2b. These show that Erythrosin B performs similarly to Rose Bengal in terms of its photobiocidal profile with pH.

Example 7

Further suspension tests were carried out as described above at pH 7 using the following solutions:
Nonionic surfactant Imbentin C91-35 (0.7%)
Imbentin C91-35 (0.7%) with Rose Bengal (100 ppm)
Anionic surfactant Empicol LX (0.7%)
Empicol LX with Rose Bengal (100 ppm)

Imbentin C91-35, Empicol LX (both 0.7%) and Rose Bengal (100 ppm).

The results are set out below in Table 5. In the Table the term "dark" indicates conditions of reduced light light exposure rather than total darkness because of the practical difficulties of avoiding some light exposure. In the Table the term "light" indicates results that are the average of several experiments carried out over a range of times (20 mins, 1 hour, 3 hours) in a statistically designed experiment.

Results obtained in similar manner for E. coli at pH 7 are shown graphically in the bar charts of Figure 4. In this Figure PAS is used as an abbreviation for Empicol LX, IPA is used as an abbreviation for isopropanol and AE as an abbreviation for Imbentin C91-35. This figure represents averaged data for AE 0.7%, PAS 0.7%, IPA 10%.

Example 8

Further suspension tests were performed at pH 4 for E. coli using one or more of the following: Rose Bengal 40ppm, surfactant 0.7% (Imbentin C91-35 or Empicol LX), IPA (isopropanol) 10%. Results are given in Table 6.

The following examples concern suspension tests carried out generally as described above, but at pH 4. Rose Bengal when used was present at a concentration of 20ppm, although in some cases control solutions without Rose Bengal were exposed to light and the results for these are given in the column headed "No Rose Bengal".

Examples 9, 10, 11 and 12 used the Gram positive organism S. aureus, and Examples 13 and 14 the Gram negative organism E. coli. Other reagents used are indicated in

the examples. In all these Examples, samples were exposed for 20 minutes on a light box. The average intensity at the surface of the diffuser was 4000 lux measured with a Megatron DA10 light meter (from Megatron Ltd).

Example 9

Suspension tests were carried out using Rose Bengal, ethanol and Imbentin C91-35, with S. aureus. The decadic logarithm of the starting concentration, log (start), of S. aureus was 6.8. Results are given in Table 7.

Example 10

Suspension tests were carried out using Rose Bengal, Dowanol PnB and Imbentin C91-35, with S. aureus. The log (start) was 6.9. Results are given in Table 8.

This example shows that Dowanol PnB has certain biocidal properties.

Example 11

Suspension tests were carried out using Rose Bengal, ethylene glycol and Imbentin C91-35, with S. aureus. The log (start) was 6.8. Results are given in Table 9.

Example 12

Suspension tests were carried out using Rose Bengal, IPA and Lialet 111, with S. aureus. Lialet 111 is the trade name of an ether sulphate formulation commercially available from Enichem, having an average chain length 11

with an average degree of ethoxylation of 3. The log (start) was 6.7. Results are given in Table 10.

Example 13

Suspension tests were carried out using Rose Bengal, propan-2-ol and Imbentin C91-35, with E. coli. The log (start) was 6.8. Results are given in Table 11.

Example 14

Suspension tests were carried out using Rose Bengal, ethanol and Imbentin C91-35, with E. coli. The log (start) was 7.1. Results are given in Table 12.

Example 15

Rose Bengal adsorption was determined from the depletion in solution concentration. Concentrations were obtained spectroscopically from absorbances measured at the wavelength of maximum absorbance (ca. 549 nm) using a WPA Linton S110 spectrophotometer on supernatant liquors freed from microbes by centifugation.

The results ar shown in Figures 5 to 8.

The results show that the photobiocidal effect is dependent on dye adsorption. Dye adsoprtion is:

a) increased by low pH (Figure 4);
b) increased by netural electrolyte (which also increases the photobiocidal effect at neutral pH on E. coli) (Figure 6);
c) decreased by surfactant (Figure 7);

d) increased by propan-2-ol (Figure 8).

Calculations show the amount adsorbed onto E. coli is of the right order of magnitude for monolayer coverage, given that dyes are known to aggregate and the calculated surface are of E. coli must be an underestimate (no account taken of fimbriae/pili). Brief details of the calculations are given below. The molecular dimensions of Rose Bengal were taken from a scale model (Catalin Ltd., London).

| | |
|---|---|
| Surface area of E. Coli | 1E7 sq.nm |
| Area of Rose Bengal | 2 sq.nm (flat)<br>0.5 sq.nm (side) |
| Monolayer coverage | 5E6 (flat) or 20E6 (side) |
| Measured adsorption | 2 - 8 E-16 Moles/bacteria |
| Number of molecules | 120 - 480 E6 per bacterium |

Example 16

Suspension tests were carried out as described above to determine the effect on the rate of kill of E. coli by Rose Bengal (20 ppm) of pH and addition of electrolyte (Na sulphate, 5%) at pH 7, and the results are shown graphically in Figures 9 and 10, respectively.

Table 1 (Example 1)

Photobiocidal Effect of Disclosing Agents
Zone of Inhibition Around Disc

| Organism | Gram Type | Rose Bengal | Erythrosin B | APS |
|---|---|---|---|---|
| S. aureus | + | 4 | 1 | 2 |
| B. subtilis | + | 3 | 1 | 1 |
| B. megaterium | + | 3 | 1 | 1.5 |
| E. coli | - | 0 | 0 | 0 |
| K. pneumoniae | - | 1.5 | 0 | 3 |
| Ps. aeruginosa | - | 0 | 0 | 0 |
| Enterobacter sp. | - | 0 | 0 | 0 |
| C. albicans | | 0 | 0 | 0 |

Table 2 (Example 2)

The Lethal Effect of Rose Bengal and Light on Staphylococcus Aureus Attached to a Plastic Surface

| Solution | Log (Ratio) |
|---|---|
| Rose Bengal | 4.7 |
| Rose Bengal + Imbentin C91-35 + Ethanol | 6.0 |

Table 3 (Example 3)

| Organism | Experiment Number | Results from Agar Overlay Technique | |
|---|---|---|---|
| | | Control | After Exposure |
| S. aureus | 1 | Confluent growth | No growth |
| | 2 | Confluent growth | No growth |
| E. coli | 1 | Much growth | 45 cfu |
| | 2 | Confluent growth | No growth |
| K. pneumoniae | 1 | Confluent growth | No growth |
| | 2 | Confluent growth | No growth |
| P. aeruginosa | 1 | Much growth | 5 cfu |
| | 2 | Confluent growth | No growth |
| C. albicans | 1 | Confluent growth | 5 cfu |
| | 2 | Confluent growth | 4 cfu |

Table 4 (Example 4)

| Organism | Gram Type | Conditions of Exposure | | | |
|---|---|---|---|---|---|
| | | No Solvent | | With Ethanol | |
| | | pH 7 | pH4 | pH 7 | pH4 |
| S. aureus | + | 7.0 | 7.1 | 7.1 | 6.9 |
| B. subtilis | + | 0.6 | 2.1 | 3.1 | 0.8 |
| B. megaterium | + | 1.3 | 0.3 | 1.1 | 0.3 |
| E. coli | - | 0.2 | 5.3 | 0.1 | 6.9 |
| K. pneumoniae | - | 0.9 | 5.6 | 0 | 7.0 |
| Ps. aeruginosa | - | 0 | 7.0 | 0 | 6.9 |
| Enterobacter sp. | - | 1.1 | 7.3 | 0 | 7.4 |
| C. albicans | | 0 | 5.7 | 0 | 5.7 |
| | | Controls (No Rose Bengal) | | | |
| E. coli | - | | 0.1 | | 0.8 |

Table 5 (Example 7)

| ORGANISM | IMBENTIN | | IMBENTIN/RB | | EMPICOL | | EMPICOL/RB | |
|---|---|---|---|---|---|---|---|---|
| | light | dark | light | dark | light | dark | light | dark |
| St. aureus | 4.5 | 4.0 | 8.0 | 3.5 | 4.5 | 4.5 | 5.5 | 5.0 |
| E. coli | 9.0 | 9.5 | 10.5 | 10.0 | 5.5 | 5.0 | 6.5 | 6.0 |
| Entero | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Klebsiella | 7.0 | 6.0 | 6.0 | 7.0 | 6.0 | 6.0 | 6.0 | 6.0 |
| Ps. aerug. | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| C. albicans | 0 | 0 | 1.0 | 1.0 | 2.5 | 1.5 | 3.0 | 3.0 |

| ORGANISM | IMBENTIN/EMPICOL | | IMBENTIN/EMPICOL/RB | | ROSE BENGAL | |
|---|---|---|---|---|---|---|
| | light | dark | light | dark | light | dark |
| St. aureus | 3.0 | 4.0 | 2.0 | 0 | 4.0 | 0 |
| E. coli | 4.0 | 4.0 | 4.0 | 4.5 | 0 | 0 |
| Entero. | 0 | 0 | 0 | 0 | 0 | 0 |
| Klebsiella | 3.5 | 3.5 | 4.0 | 2.5 | 0 | 0 |
| Ps. aerug. | 0 | 0 | 0 | 0 | 0 | 0 |
| C. albicans | 0 | 0 | 0 | 0 | 0 | 0 |

Table 6 (Example 8)

| | EXPOSURE TIME | | | | | |
|---|---|---|---|---|---|---|
| | 1 HOUR | | 2 HOURS | | 3 HOURS | |
| | light | dark | light | dark | light | dark |
| Rose Bengal | 3.8 | 0.5 | 7.2 | 0.4 | 7.2 | 0.7 |
| RB/Imbentin C91-35 | 0.2 | 0.2 | 1.0 | 0.3 | 3.8 | 0.5 |
| RB/Empicol LX | 1.7 | 0.4 | 3.9 | 0.7 | 7.2 | 0.7 |
| RB/IPA | 5.4 | 3.6 | 4.7 | 5.0 | 7.2 | 7.2 |
| Imbentin C91-35 | 0.1 | | 0.3 | | 0.9 | |
| Empicol LX | 0.1 | | 0.6 | | 1.0 | |
| IPA | 0.3 | | 1.7 | | 4.4 | |

Table 7 (Example 9)

| Ethanol % | Imbentin C91-35 % | Log (reduction) After Light Exposure | Log (reduction) No Rose Bengal |
|---|---|---|---|
| 5 | 0.2 | +6.8 | |
| 5 | 0.6 | 4.6 | |
| 10 | 0.6 | +6.8 | |
| 15 | 0.6 | +6.8 | |
| 5 | - | +6.8 | 0.1 |
| 10 | - | +6.8 | -0.4 |
| 15 | - | +6.8 | 0.0 |
| - | 0.2 | 4.6 | 2.5 |
| - | 0.6 | 3.5 | 2.3 |
| - | - | +6.8 | 2.3 |

Table 8 (Example 10)

| Dowanol % | Imbentin C91-35 % | Log (reduction) After Light Exposure | Log (reduction) No Rose Bengal |
|---|---|---|---|
| 3 | 0.7 | +6.9 | |
| 3 | - | +6.9 | 4.0 |
| - | 0.7 | 5.2 | 3.4 |
| - | - | +6.9 | |

Table 9 (Example 11)

| Ethylene Glycol % | Imbentin C91-35 % | Log (reduction) After Light Exposure | Log (reduction) No Rose Bengal |
|---|---|---|---|
| 10 | 0.7 | +6.8 | |
| 10 | - | +6.9 | -0.2 |
| - | 0.7 | +6.8 | 3.4 |
| - | - | +6.8 | |

Table 10 (Example 12)

| Propan-2-ol % | Lialet 111 % | Log (reduction) After Light Exposure | Log (reduction) No Rose Bengal |
|---|---|---|---|
| 15 | 0.5 | +6.7 | |
| 15 | - | +6.7 | 4.9 |
| - | 0.5 | +6.7 | +6.7 |
| - | - | +6.7 | |

Table 11 (Example 13)

| Propan-2-ol % | Imbentin C91-35 % | Log (reduction) After Light Exposure | Log (reduction) No Rose Bengal |
|---|---|---|---|
| 5 | 0.1 | 2.3 | |
| 10 | 0.1 | +6.8 | |
| 10 | 0.5 | +6.8 | |
| 10 | 0.7 | +6.8 | |
| - | - | 4.8 | |
| 5 | - | 5.5 | 0.3 |
| 10 | - | 3.0 | 1.9 |
| - | 0.1 | 1.2 | 1.3 |
| - | 0.5 | 1.0 | 1.2 |
| - | 0.7 | 1.1 | 1.1 |

Table 12 (Example 14)

| Ethanol % | Imbentin C91-35 % | Log (reduction) After Light Exposure | Log (reduction) No Rose Bengal |
|---|---|---|---|
| 5 | 0.2 | 4.1 | |
| 15 | 0.6 | +7.1 | |
| 5 | - | 5.0 | 0.2 |
| 10 | - | +7.1 | 0.2 |
| 15 | - | +7.1 | 2.2 |
| - | 0.2 | 3.3 | 2.5 |
| - | 0.6 | 3.4 | 2.6 |
| - | - | 3.9 | |

CLAIMS

1. A surface germicidal composition comprising a dyestuff which is capable of photo-dynamic inactivation of micro-organisms.

2. A composition according to claim 1, wherein the dyestuff generates singlet oxygen an exposure to light.

3. A composition according to claim 1 or 2, wherein the dyestuff is substantive to micro-organisms.

4. A composition according to claim 1, 2 or 3, wherein the dyestuff is bleached by exposure to light.

5. A composition according to any one of the preceding claims, wherein the dyestuff is selected from the group comprising Rose Bengal, Erythrosin B and phthalocyanin sulphonates.

6. A composition according to any one of the preceding claims, wherein dyestuff is present in an amount in the range 1 to 100 ppm.

7. A composition according to any one of the preceding claims, further comprising one or more surfactants.

8. A compositon according to claim 7, wherein the surfactant is alkoxylated.

9. A composition according to claim 8, wherein the surfactant is ethoxylated.

10. A composition according to claim 7, 8 or 9, wherein the surfactant is at least predominantly non-ionic and/or anionic.

11. A composition according to any one of claims 7 to 10, wherein surfactant is present in an amount in the range 0.05 to 2.5% by weight of the total weight of the composition.

12. A composition according to any one of the preceding claims, further comprising one or more solvents.

13. A composition according to claim 12, wherein the solvent is polar.

14. A composition according to claim 13, wherein the solvent is a straight or branched chain C2 to C5 alcohol.

15. A composition according to claim 12, 13 or 14, wherein solvent is present in an amount in the range 2 to 20% by weight of the total weight of the composition.

16. A composition according to any one of the preceding claims, having a pH in the range 3 to 5.

17. A composition according to claim 16, having a pH of about 4.

18. A surface cleaning and germicidal composition, comprising a dyestuff which is capable of photo-dynamic inactivation of micro-organisms, a surfactant and a solvent.

19. A method of killing bacteria on a surface, comprising

applying to the surface a composition in accordance with any one of the preceding claims.

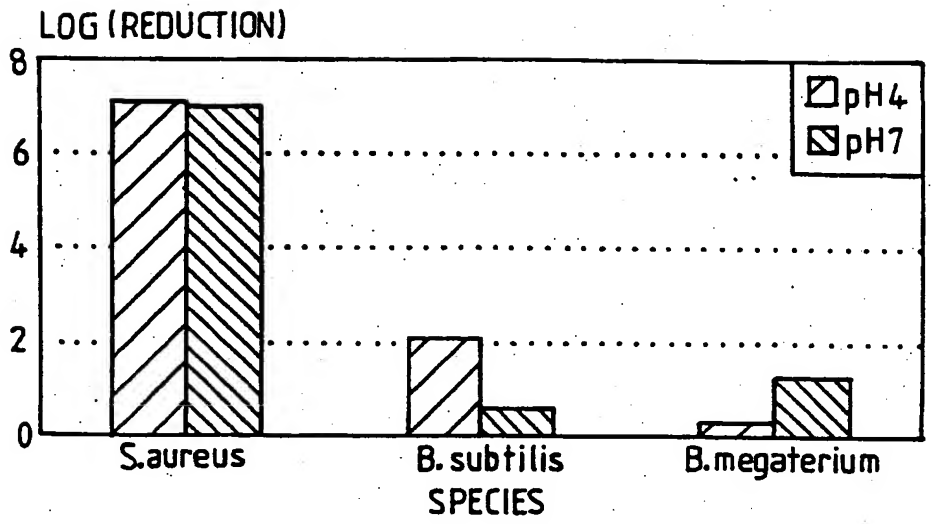
LOG (REDUCTION)
8
6
4
2
0
pH4
pH7
S.aureus
B. subtilis
B.megaterium
SPECIES

Fig. 1a

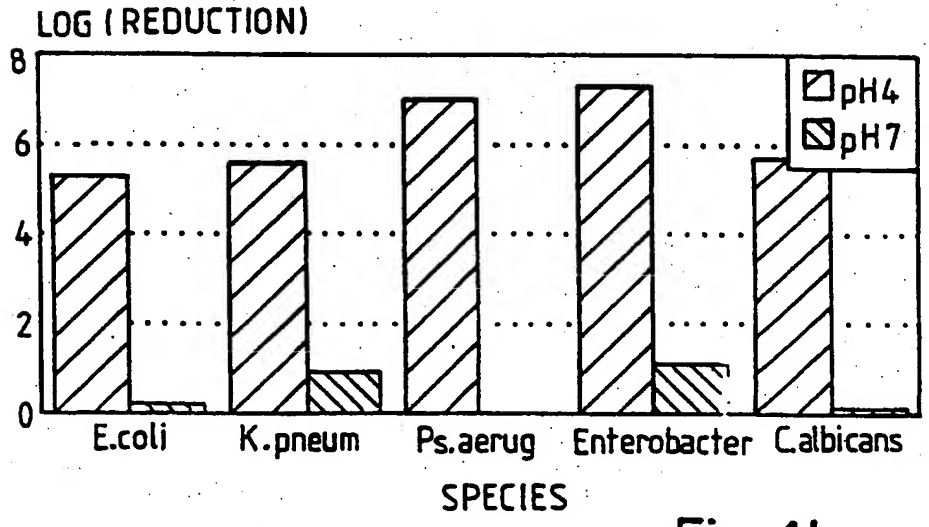
LOG (REDUCTION)
8
6
4
2
0
pH4
pH7
E.coli
K.pneum
Ps.aerug
Enterobacter
C.albicans
SPECIES

Fig. 1b

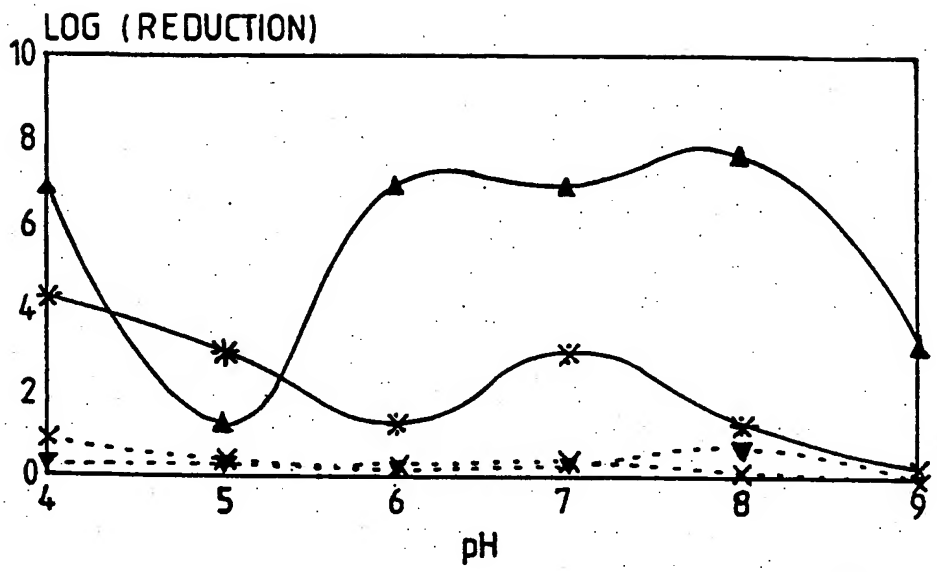
LOG (REDUCTION)
10
8
6
4
2
0
4
5
6
7
8
9
pH

Fig 2a

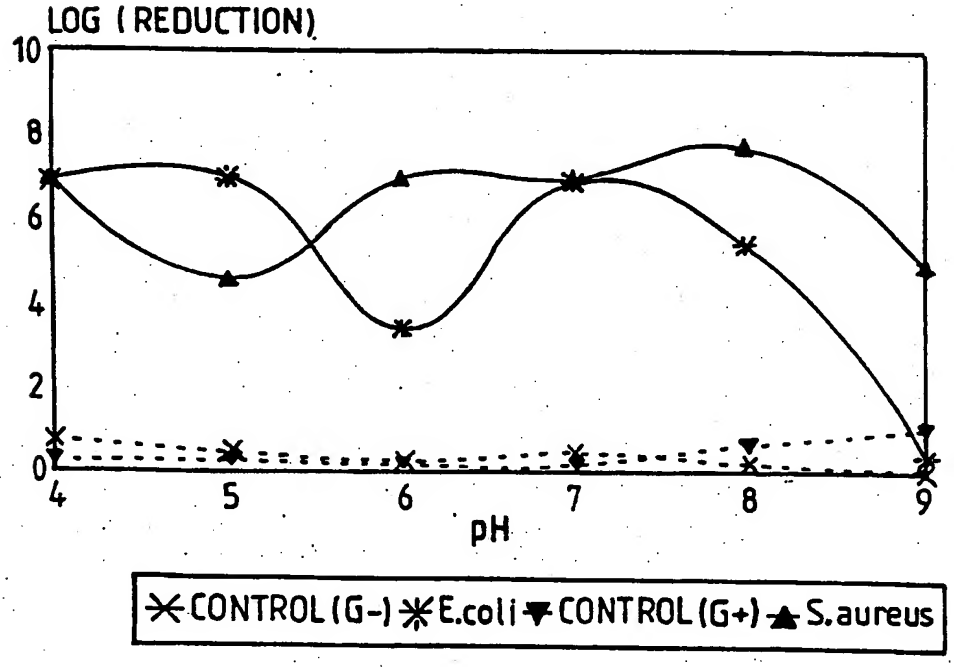
LOG (REDUCTION)
10
8
6
4
2
0
4
5
6
7
8
9
pH
CONTROL (G-)
E.coli
CONTROL (G+)
S.aureus

Fig. 2b

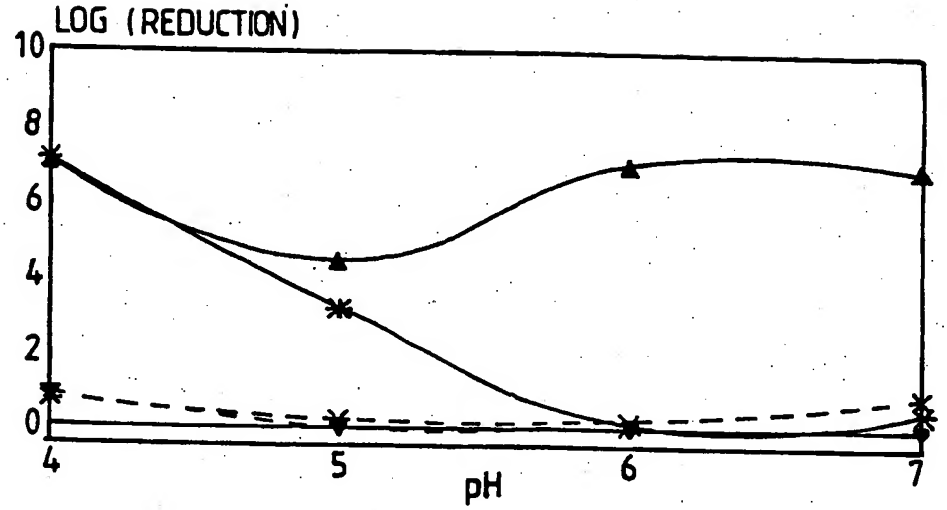
LOG (REDUCTION)
10
8
6
4
2
0
4
5
6
7
pH

Fig. 3a

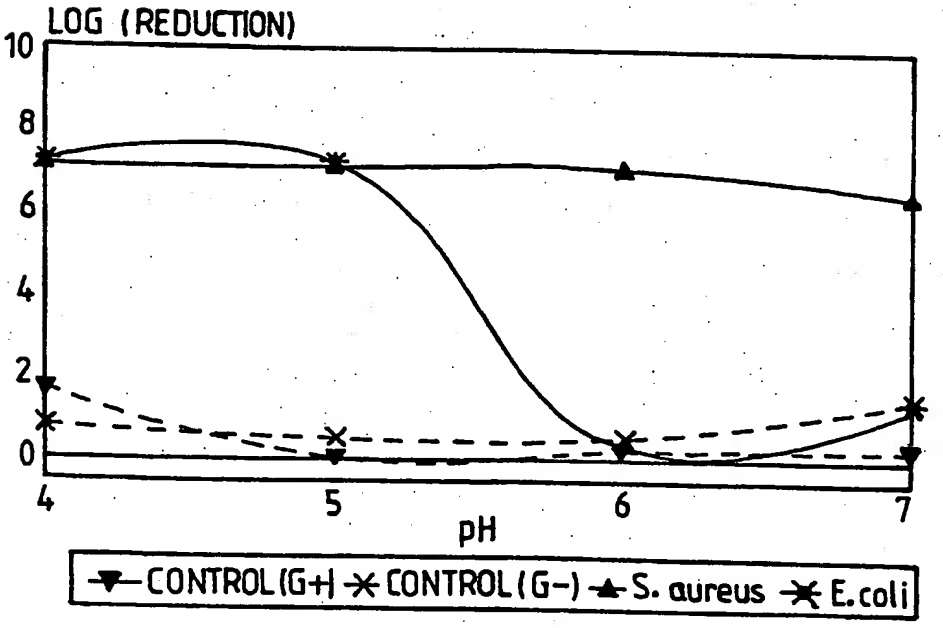
LOG (REDUCTION)
10
8
6
4
2
0
4
5
6
7
pH
CONTROL (G+)
CONTROL (G-)
S. aureus
E. coli

Fig. 3b

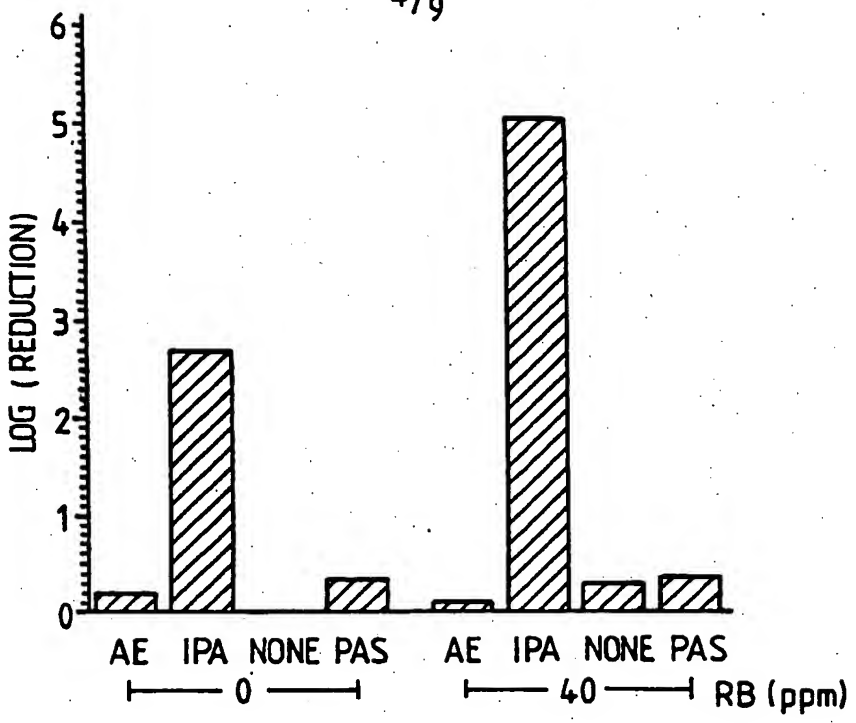
LOG (REDUCTION)
0
1
2
3
4
5
6
AE IPA NONE PAS AE IPA NONE PAS
0
40
RB (ppm)

Fig. 4a

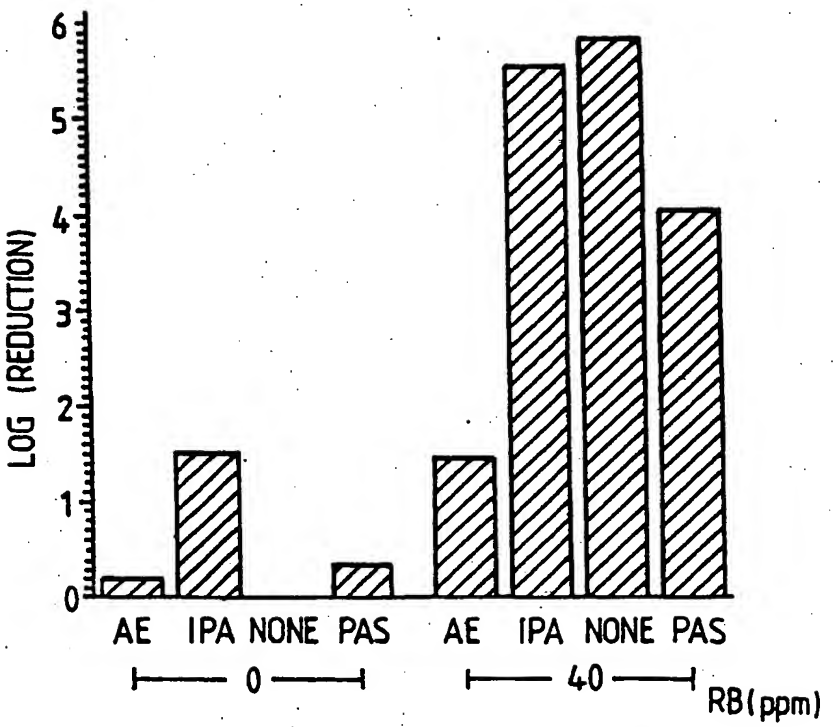
LOG (REDUCTION)
0
1
2
3
4
5
6
AE IPA NONE PAS AE IPA NONE PAS
0
40
RB(ppm)

Fig. 4b

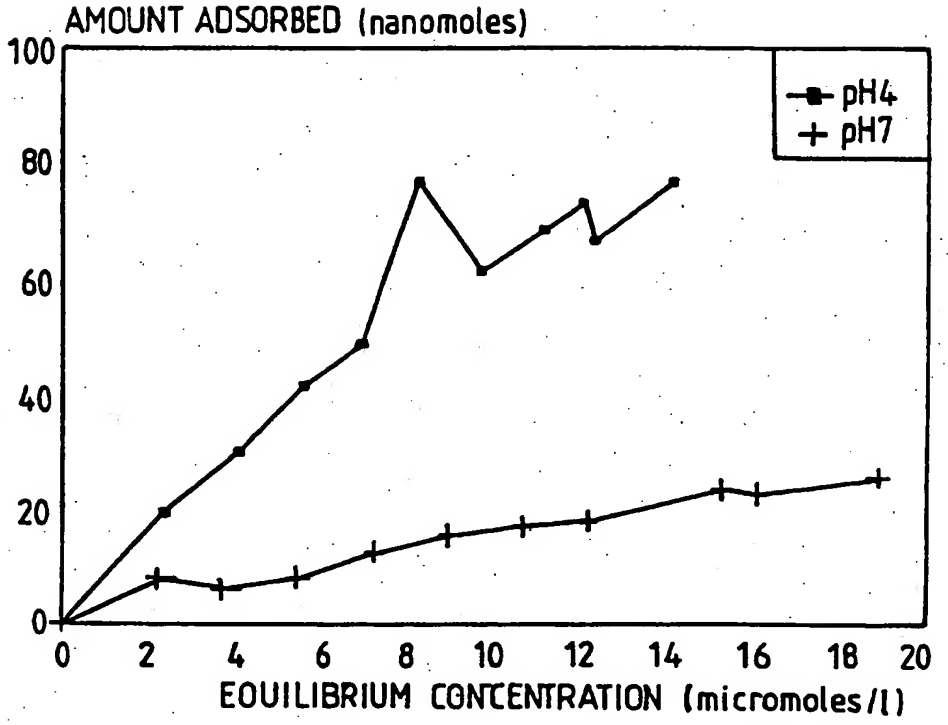
AMOUNT ADSORBED (nanomoles)
100
80
60
40
20
0
0
2
4
6
8
10
12
14
16
18
20
EQUILIBRIUM CONCENTRATION (micromoles/l)
pH4
pH7

Fig. 5

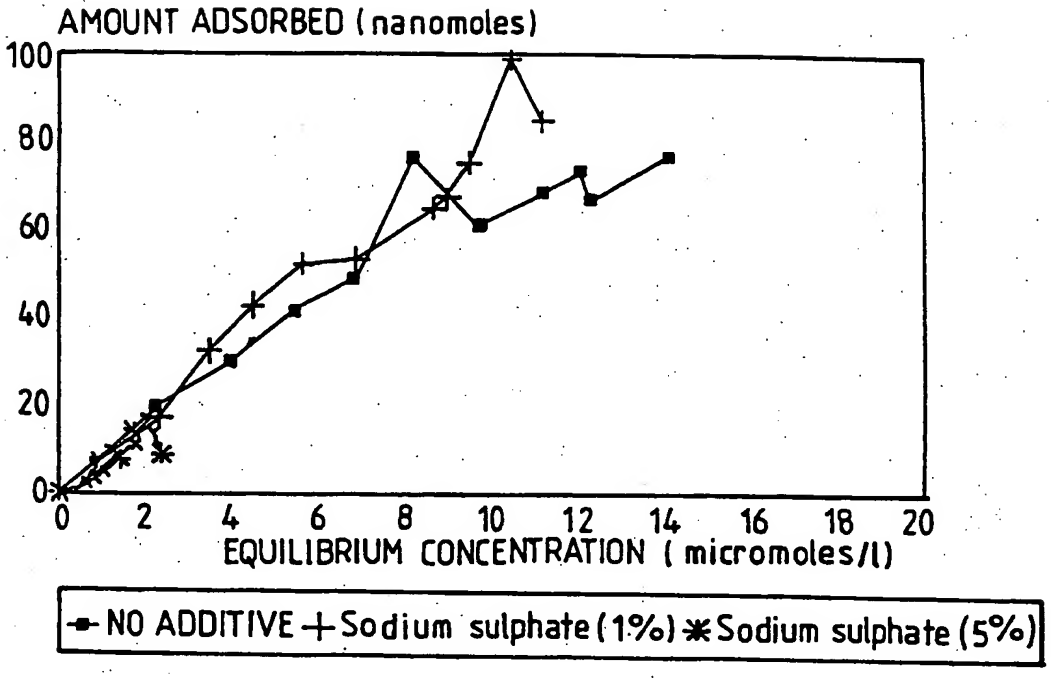
AMOUNT ADSORBED (nanomoles)
100
80
60
40
20
0
0 2 4 6 8 10 12 14 16 18 20
EQUILIBRIUM CONCENTRATION ( micromoles/l)
NO ADDITIVE
Sodium sulphate (1%)
Sodium sulphate (5%)

Fig. 6a

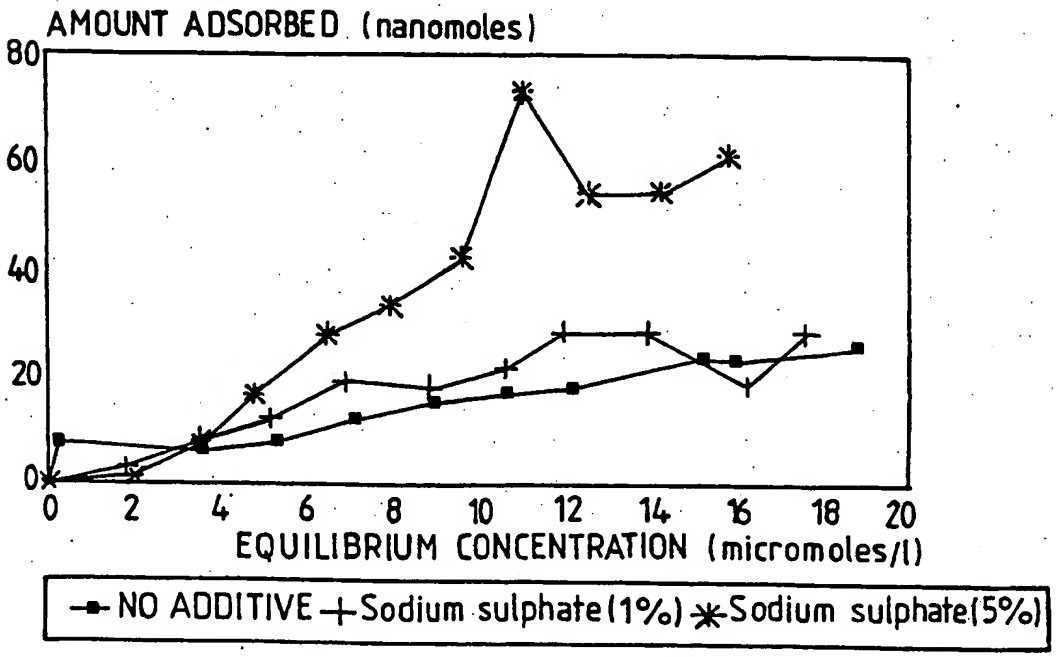
AMOUNT ADSORBED (nanomoles)
80
60
40
20
0
0 2 4 6 8 10 12 14 16 18 20
EQUILIBRIUM CONCENTRATION (micromoles/l)
NO ADDITIVE
Sodium sulphate(1%)
Sodium sulphate(5%)

Fig. 6b

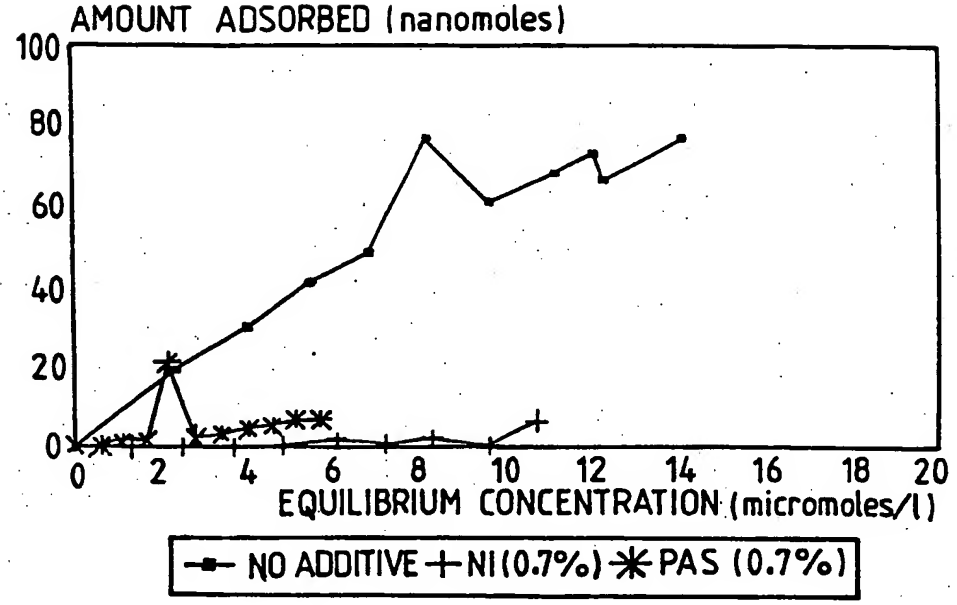
AMOUNT ADSORBED (nanomoles)
100
80
60
40
20
0
0 2 4 6 8 10 12 14 16 18 20
EQUILIBRIUM CONCENTRATION (micromoles/l)
NO ADDITIVE
NI (0.7%)
PAS (0.7%)

Fig. 7a

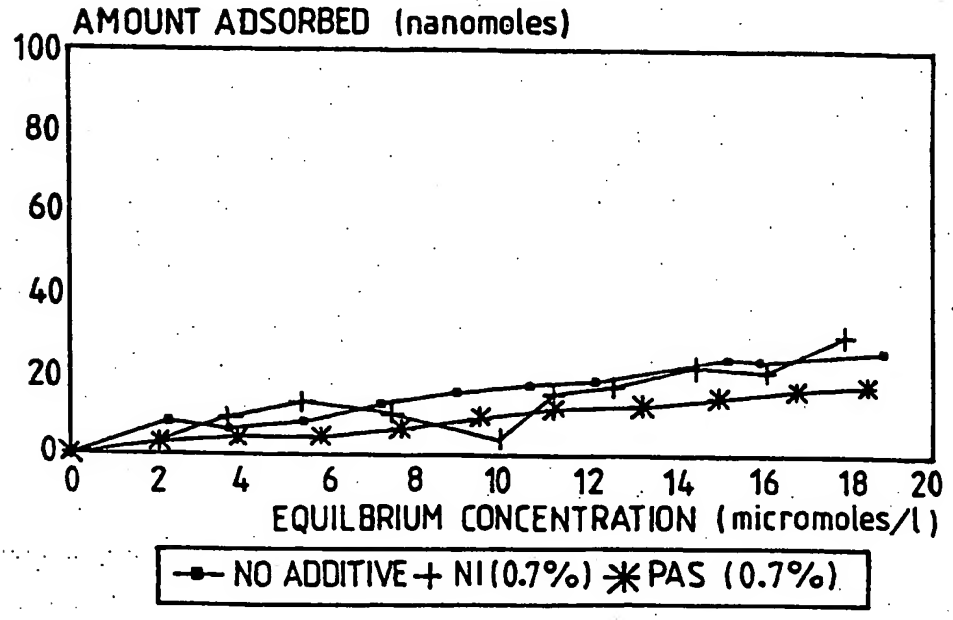
AMOUNT ADSORBED (nanomoles)
100
80
60
40
20
0
0 2 4 6 8 10 12 14 16 18 20
EQUILBRIUM CONCENTRATION (micromoles/l)
NO ADDITIVE
NI (0.7%)
PAS (0.7%)

Fig. 7b

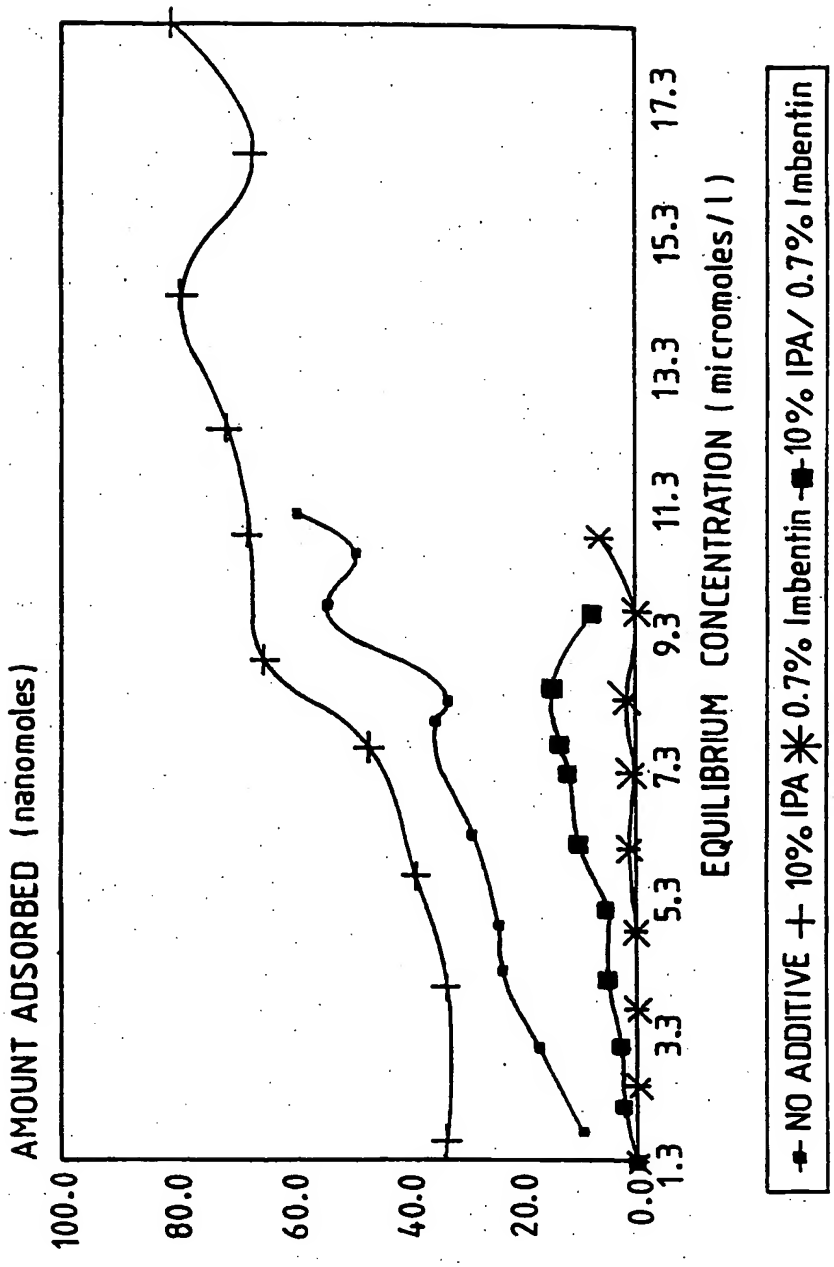
AMOUNT ADSORBED (nanomoles)
100.0
80.0
60.0
40.0
20.0
0.0
1.3
3.3
5.3
7.3
9.3
11.3
13.3
15.3
17.3
EQUILIBRIUM CONCENTRATION (micromoles/l)
NO ADDITIVE
10% IPA
0.7% Imbentin
10% IPA/ 0.7% Imbentin

Fig. 8

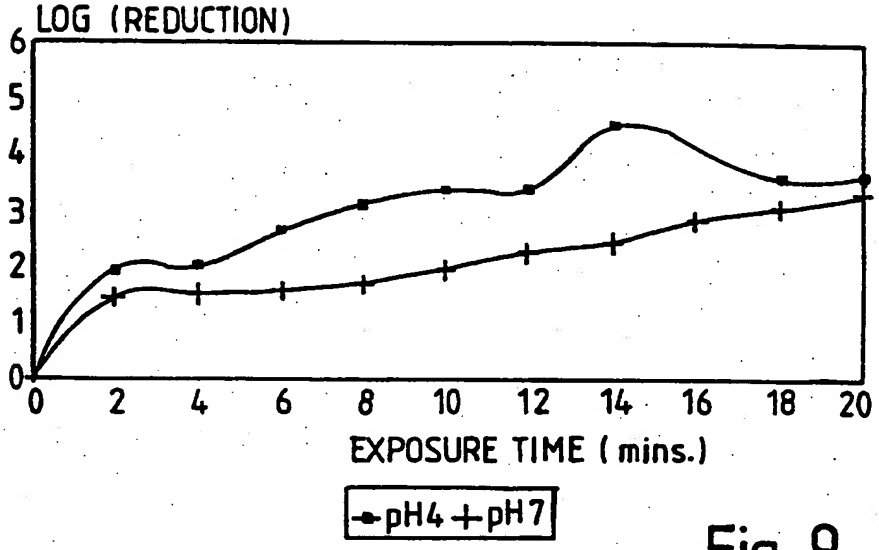
LOG (REDUCTION)
0
1
2
3
4
5
6
0 2 4 6 8 10 12 14 16 18 20
EXPOSURE TIME ( mins.)
pH4 + pH7

Fig. 9

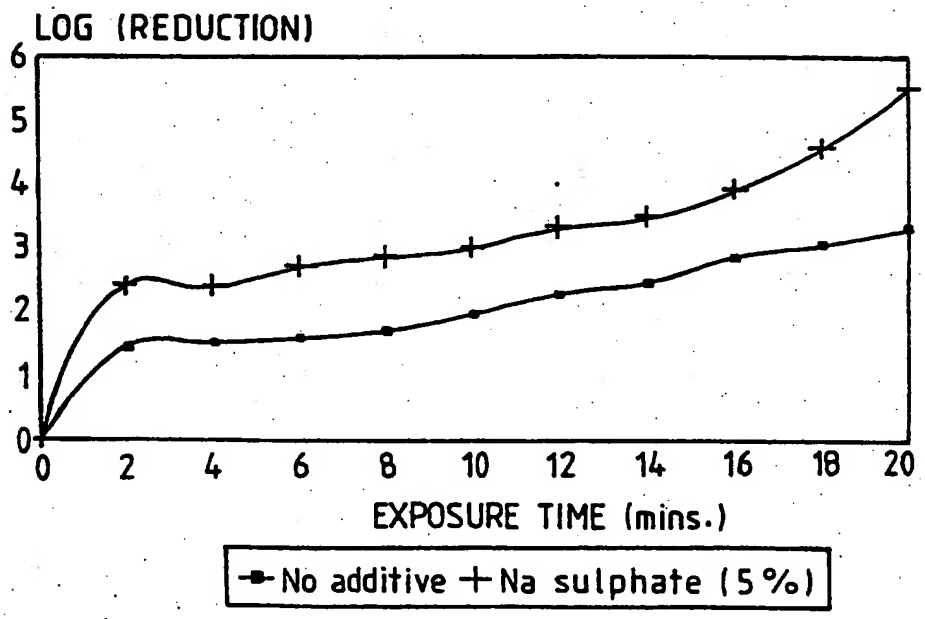
LOG (REDUCTION)
0
1
2
3
4
5
6
0 2 4 6 8 10 12 14 16 18 20
EXPOSURE TIME (mins.)
No additive + Na sulphate (5%)

Fig. 10

# INTERNATIONAL SEARCH REPORT

Inter nal Application No
PCT/GB 93/01478

A. CLASSIFICATION OF SUBJECT MATTER
IPC 5 A01N61/00 A01N43/90 A01N43/16

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 5 A01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

| Category * | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| X | EP,A,0 081 462 (CIBA-GEIGY) 15 June 1983<br>see page 1, paragraph 1<br>see page 13, paragraph 5<br>see page 16, paragraph 2 -paragraph 3<br>see page 17, paragraph 2 -paragraph 4<br>see page 19, paragraph 3 - page 20, paragraph 1<br>see page 21, paragraph 1 - page 23, paragraph 1<br>see page 36; example 9<br>--- | 1-19 |
| X,Y | DE,A,28 12 261 (CIBA-GEIGY) 28 September 1978<br>see claims 1,17<br>see page 18, paragraph 1<br>see page 19, paragraph 1<br>---<br>-/-- | 1-19 |

[X] Further documents are listed in the continuation of box C.

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# INTERNATIONAL SEARCH REPORT

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

| Category * | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| Y | DATABASE CHEMABS<br>CHEMICAL ABSTRACTS SERVICE, COLUMBUS, OHIO, US<br>AN: CA74(21):108555b<br>K.INADA ET AL. 'Relation between photodynamic activities and photochemical oxidation in xanthene dyes'<br>see abstract<br>& SHOKUHIN EISEIGAKU ZASSHI<br>vol. 11, no. 4 , 1970<br>pages 238 - 241 | 1-19 |
| X | EP,A,0 286 524 (B.B.C) 12 October 1988<br>see column 1, line 51 - column 2, line 2<br>see column 3, line 24 - line 29 | 1-6,12, 13,19 |
| X | DATABASE BIOSIS<br>BIOSCIENCES INFORMATION SERVICE, PHILADELPHIA, PA, US<br>AN: 88:25633; DN: BA85:13358<br>M.I.ROAT 'THE ANTIVIRAL EFFECTS OF ROSE BENGAL AND FLUORESCEIN'<br>see abstract<br>& ARCH OPHTHALMOL<br>vol. 105, no. 10 , 1987<br>pages 1415 - 1417 | 1-6,19 |
| A | DATABASE CHEMABS<br>CHEMICAL ABSTRACTS SERVICE, COLUMBUS, OHIO, US<br>AN: CA72(19):97667v<br>K.INADA ET AL. 'Photodynamic action of food dyes on bacteria'<br>see abstract<br>& SHOKUHIN EISEIGAKU ZASSHI<br>vol. 10, no. 5 , 1969<br>pages 344 - 347 | 1-19 |
| A | DATABASE CHEMABS<br>CHEMICAL ABSTRACTS SERVICE, COLUMBUS, OHIO, US<br>AN: CA90(17):134960e<br>S.A.BEZMAN ET AL. 'Photodynamic inactivation of E. coli by rose bengal immobilized on polystyrene beads'<br>see abstract<br>& PHOTOCHEM. PHOTOBIOL.<br>vol. 28, no. 3 , 1978<br>pages 325 - 329 | 1-19 |

# INTERNATIONAL SEARCH REPORT

Information on patent family members

Inter nal Application No
PCT/GB 93/01478

| Patent document cited in search report | Publication date | Patent family member(s) | | Publication date |
|---|---|---|---|---|
| EP-A-0081462 | 15-06-83 | AU-A- | 9133482 | 16-06-83 |
| | | CA-A- | 1201711 | 11-03-86 |
| | | GB-A,B | 2113677 | 10-08-83 |
| | | JP-C- | 1745848 | 25-03-93 |
| | | JP-B- | 4028714 | 15-05-92 |
| | | JP-A- | 58105989 | 24-06-83 |
| | | JP-A- | 5017485 | 26-01-93 |
| | | US-A- | 4497741 | 05-02-85 |
| | | US-A- | 4566874 | 28-01-86 |
| DE-A-2812261 | 28-09-78 | CH-A- | 640238 | 30-12-83 |
| | | AT-B- | 361637 | 25-03-81 |
| | | AU-A- | 3475778 | 11-10-79 |
| | | BE-A- | 865291 | 25-09-78 |
| | | CA-A- | 1138442 | 28-12-82 |
| | | FR-A,B | 2387658 | 17-11-78 |
| | | FR-A,B | 2387985 | 17-11-78 |
| | | GB-A- | 1593623 | 22-07-81 |
| | | GB-A- | 1593624 | 22-07-81 |
| | | JP-C- | 1705632 | 27-10-92 |
| | | JP-B- | 3071401 | 13-11-91 |
| | | JP-A- | 53119884 | 19-10-78 |
| | | JP-A- | 2000181 | 05-01-90 |
| | | JP-B- | 5015714 | 02-03-93 |
| | | JP-A- | 1279807 | 10-11-89 |
| | | JP-B- | 4077040 | 07-12-92 |
| | | NL-A- | 7803095 | 27-09-78 |
| | | US-A- | 4318883 | 09-03-82 |
| | | US-A- | 4530924 | 23-07-85 |
| EP-A-0286524 | 12-10-88 | FR-A- | 2613626 | 14-10-88 |
| | | DE-A- | 3875511 | 03-12-92 |
| | | JP-A- | 63264064 | 31-10-88 |
| | | OA-A- | 8828 | 31-03-89 |